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The pH dependency of N-converting enzymatic processes, pathways and microbes: effect on net-N_2O production

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Summary

Nitrous oxide (N_2O) is emitted during microbiological nitrogen (N) conversion processes, when N_2O production exceeds N_2O consumption. The magnitude of N_2O production vs consumption varies with pH and controlling net-N_2O production might be feasible by choice of system pH. This article reviews how pH affects enzymes, pathways and microorganisms that are involved in N-conversions in water engineering applications. At a molecular level, pH affects activity of co-factors and structural elements of relevant enzymes, by protonation or deprotonation of amino acid residues or solvent ligands causing steric changes in catalytic sites or proton/electron transfer routes that alter catalytic activity. Augmenting molecular information with, e.g. nitritation or denitrification rates, yields explanations of net-N_2O production with pH changes. Ammonia oxidizing

bacteria are of highest relevance for N_2O production, while heterotrophic denitrifiers are relevant for N_2O consumption at pH > 7.5. Net-N_2O production in N-cycling water engineering systems is predicted to display a “bell-shaped” curve in the range of pH 6.0-9.0 with a maximum at pH 7.0-7.5. Net N_2O production at acidic pH is dominated by N_2O production, whereas N_2O consumption can outweigh production at alkaline pH. Thus, pH 8.0 may be a favorable pH set-point for water treatment applications regarding net N_2O production.

1. Introduction

Emissions of nitrous oxide (N_2O) to the atmosphere are of concern, as N_2O is a greenhouse gas with a large global warming potential and ozone depleting properties (Ravishankara *et al.*, 2009; IPCC, 2013). As a result of an increased load of reactive nitrogen (N) to the biological N-cycle by anthropogenic activities, emissions of N_2O are increasing (Canfield *et al.*, 2010; Steffen *et al.*, 2015). Especially agricultural soils and engineered nitrogen removal systems, like constructed wetlands or urban wastewater treatment plants (WWTPs), constitute hubs of N_2O emissions (Canfield *et al.*, 2010; Law *et al.*, 2012). Nitrogen is introduced to these systems in its most reduced form, i.e. as ammonium (NH_4^+) or organic nitrogen (which is readily converted to ammonium via ammonification). Whilst in agriculture the assimilation of NH_4^+-N into biomass is the primary objective, water treatment applications seek to convert NH_4^+ into environmentally inert dinitrogen gas (N_2). In all systems the loss of nitrogen in the form of N_2O is significant and strategies to mitigate N_2O emissions are sought after (Hénault *et al.*, 2012; Law *et al.*, 2012).

Various prokaryotes harbor the energy associated with conversion of N-species between different redox states. The biological N-network hosts a multitude of conversion reactions, which are catalyzed by a variety of enzymes (Fig. 1). N_2O constitutes the end product of a number of metabolic pathways or is an intermediate towards N_2. As, in principle, both N_2O production and consumption reactions exist, net-N_2O production occurs as the result of non-ideal flow of N-species through the N-network (Stein, 2010). Hence, the key to lower N_2O emissions are overall pathways that prevent the accumulation of N_2O. pH is one of the parameters that affect conversion rates of enzymes (Illanes *et al.*, 2008). The enzymes involved in the N-network have different pH optima and pH may cause imbalances

between enzymatic reaction steps that lead to the accumulation of intermediates, such as hydroxylamine (NH_2OH), nitrite (NO_2^-), nitric oxide (NO) or N_2O. Conversely, setting pH may offer an opportunity to synchronize N-conversion rates to reduce accumulation of N_2O. A better understanding of the effect of pH on the N-network can support decision making on pH set-point management to lower N_2O emissions from soils and engineered N-removal systems.

Figure 1

This article reviews how the pH affects enzymes, pathways and microbes that are involved in the N-network and which are relevant for net-N_2O production. It further explores, if pH optima of individual enzymes hold sufficient information to hypothesize favorable pH set-points for lower net-N_2O production. Written with the background of biological N-removal during wastewater treatment processes, most examples are taken from WWTPs. However, as the same microbial pathways are active in other environments, findings may be relevant for fields outside of wastewater treatment. Microorganisms in WWTPs are usually not exposed to environmental pH values outside the range pH 6.0-9.0 (Henze and Comeau, 2008). Therefore this article focuses on effects of pH in this range.

Summarizing, the effect of pH is complex, partly because of the sheer number of enzymes, pathways and organisms involved in N-conversion, but also because pH affects various central processes in cells, signaling or transcriptional and post-transcriptional phenomena. Accordingly, the attempt to infer optimal pH set-points for complex microbial communities and their N- conversion processes with respect to N_2O is ambitious. Yet, the demand for N_2O mitigation strategies and guidelines, especially in water engineering applications, justifies an effort, even though simplifications and constraints are unavoidable. Because

such applications often involve well-controlled systems with relatively steady conditions (bioreactors), compared to rapidly changing environments of natural systems, it is plausible that pH control strategies may constitute a feasible tool to manage N_2O net production.

1.1. The effect of pH on enzymatic conversion rates

The conversion rate of an enzyme ($v=k_{cat}*[E]_0*[S]/(K_M+[S])$) is affected by pH either by changes of the turnover number (k_{cat}), the substrate concentration [S] or both (Illanes *et al.*, 2008). During nitrogen removal, pH governs the speciation of the acid/base pairs NH_4^+/NH_3 (pKa=9.3) and HNO_2/NO_2^- (pKa=3.4) (Nelson and Cox, 2005). Substrate speciation becomes relevant, when it changes the availability of substrate for enzymatic reactions or causes inhibition. k_{cat} is affected by pH, when changes of the enzymatic structure reduce the catalytic activity of enzymes (Illanes *et al.*, 2008). Based on the interplay of k_{cat} and the substrate concentration, pH set-points may be used to manage one or the other in order to control enzymatic conversion rates.

1.2. The relevance of the cellular location of enzymes for an effect of changes in ambient pH: periplasmic pH and cytoplasmic pH homeostasis in bacteria and archaea

To manage k_{cat} by pH set-points, the enzymes need to be accessible for changes of ambient pH. However, changes of pH in the environment of a cell do not directly transfer into changes of pH throughout the cell, but affect cellular compartments differently (Slonczewski *et al.*, 2009). Hence, the effect of ambient pH on the activity of an enzyme depends on its cellular location (Fig. 2). Bacteria and archaea depend on a stable pH in their cytoplasm for their metabolic machinery to operate efficiently and the proton motive force to generate chemical energy reliably (Booth, 1985). Microorganisms have developed

regulation mechanisms to maintain a near-constant cytoplasmic pH in environments with substantially different ambient pH values (Booth, 1985; Slonczewski, 2009; Krulwich, 2011). Most neutrophilic bacteria maintain their cytoplasmic pH stably at 7.5-7.7 against a pH in the environment ranging from 5.5 to 9.0 (Pedan, 2005; Slonczewski, 2009). Accordingly, a change of ambient pH in the range of pH 6.0-9.0, as present in WWTPs, would not transfer into changes of pH in the cytoplasm. Consequently, enzymes that are localized in the cytoplasm or other intra-cell compartments would not be directly affected by changes of pH in the environment (Slonczewski *et al.*, 2009; van der Star *et al.*, 2010). In contrast, the pH of the periplasm of gram-negative bacteria is not maintained and follows the pH of the environment (Wilks, 2007).

Figure 2

2. Physiological pH values of relevant prokaryotes

Physiological pH optima of microorganism combine various effects of pH on the organisms, e.g. the effect of pH on individual enzymes, the speciation of substrates and gene expression. Considerations of potential pH set-points for e.g. water treatment systems are confined by physiologically feasible pH values of microorganisms. In the following, relevant microorganisms of the N-network are introduced and physiological pH optima are summarized.

Figure 3

Aerobic ammonia oxidizing bacteria

Aerobic ammonia oxidizing bacteria (AOB) are chemolithoautotrophs that oxidize NH_3 to NO_2^- via NH_2OH, as well as NO, which has recently been recognized as another obligate

intermediate during nitritation (Caranto and Lancaster, 2017). AOB are also able to reduce NO_2^- to N_2O by the nitrifier denitrification pathway (Wrage *et al.*, 2001). The enzymes involved are ammonia monooxygenase (AMO), hydroxylamine dehydrogenase (HAO), nitrite reductase (NIR) and nitric oxide reductase (NOR) (Schreiber *et al.*, 2012). Nitritation and nitrifier denitrification play central roles in WWTPs, also in respect to N_2O production (Stein, 2011). AOB found in WWTP mainly affiliate with *Nitrosomonas*, *Nitrosospira*, *Nitrosovibrio* and *Nitrosococcus* genera, with *Nitrosomonas* and *Nitrosospira* as the most dominant AOB (Kowalchuk and Stephen, 2001). In general, AOB in WWTPs can grow across a wide range of pH with optima at pH 7.4-8.2 (Grunditz and Dalhammar, 2001; Park *et al.*, 2007; Claros *et al.*, 2013; Daalkhaijav and Nemati, 2014). Bacterial *amoA*, which is commonly used as a molecular marker for AOB, showed low abundance under acidic conditions (pH < 5.5) (De Boer and Kowalchuk, 2001) and increased with increasing pH (Leininger *et al.*, 2006; Nicol *et al.*, 2008).

Ammonia oxidizing archaea

Ammonia oxidizing archaea (AOA) perform autotrophic ammonia oxidation similar, but not identical to AOB (Kozlowski *et al.*, 2016); no HAO or NOR genes have been identified in pure or enrichment cultures of AOA, indicating that they might be unable to produce N_2O enzymatically through side reactions of ammonia oxidation or nitrifier denitrification (Walker *et al.*, 2010; Tourna *et al.*, 2011; Spang *et al.*, 2012; Stieglmeier *et al.*, 2014). AOA are widespread in nitrifying bioreactors in WWTPs or biofilters for drinking water production and are also found in soil and sediments (Park *et al.*, 2006; Zhang *et al.*, 2009; Gülay *et al.*, 2014). Optimal growth of AOA has been reported in the range of pH 7-7.5 (French *et al.*, 2012; Spang *et al.*, 2012). Nicol *et al.* (2008) observed that transcriptional abundance and expression of archaeal *amoA,* a widely used molecular marker of AOA,

decreased with increasing pH. Yet, archaeal *amoA* was found in environments with a wide range of pH (pH 3.7 to 8.65) (Erguder *et al.*, 2009). AOA have been frequently found to outnumber AOB and dominate in abundance and ammonia oxidizing activity, especially in acidic soils (Nicol *et al.*, 2008; Gubry-Rangin *et al.*, 2010; Zhang *et al.*, 2012; Li *et al.*, 2018).

Nitrite oxidizing bacteria

Nitrite oxidizing bacteria (NOB) are aerobic chemolithoautotrophs that oxidize NO_2^- to nitrate (NO_3^-). The reaction is catalyzed by the enzyme nitrite oxidoreductase (NXR) (Schreiber *et al.*, 2012). NOB have been found in several genera distributed among different bacterial lineages, i.e. *Nitrobacter*, *Nitricoccus*, *Nitrispina*, *Nitrispira* and *Nitrotoga*. Even though the genus *Nitrospira* is often the numerically dominant NOB in WWTPs (Daims *et al.*, 2001), virtually all knowledge of the physiology and biochemistry of NO_2^- oxidation has been derived from studies of a limited number of strains of *Nitrobacter* species (Daims *et al.*, 2016). The activity of NOB in WWTPs was found to be pH-dependent with optima at pH 7.9 ± 0.4 (Park *et al.*, 2007). More specifically, the optimum growth of isolated pure cultures of *Nitrospira* and *Nitrobacter* species occurred at pH 7.6-8.0 (Ehrich *et al.*, 1995) and 7.9 (Grunditz and Dalhammar, 2001), respectively.

Complete ammonia oxidizing bacteria

Complete ammonia oxidizing bacteria (Comammox) are able to oxidize NH_3 to NO_3^- (Daims *et al.*, 2015; van Kessel *et al.*, 2015). Besides NXR, *Nitrospira* sp. harbor the enzymes needed to oxidize ammonia to nitrite (AMO and HAO), which differ from the canonical enzymes utilized by proteobacterial AOB (e.g., Palomo *et al* (2016), reported only 56% similarity between amino acid sequence for *amoA* from *Nitrosomonas* and

177 *Nitrospira* sp.). AMO and NXR are membrane bound, whilst HAO is located in the
178 periplasm (Daims *et al.*, 2015). It is still to be determined, if an effect of pH on these
179 enzymes is similar to its effect on canonical AOB enzymes. Nevertheless, some studies
180 already demonstrate that pH has an impact on niche differentiation between different
181 microorganisms capable of ammonia oxidation in soils (Hu and He, 2017). *NirK*, the gene
182 for Cu-NIR, is also present in comammox, but no evidence of NO accumulation in
183 comammox enrichments cultures suggests that nitrite reduction may not be particularly
184 robust (Daims *et al.*, 2015). Comammox have been found in different water engineered
185 systems, including WWTPs (Chao *et al.*, 2016; Gonzalez-Martinez *et al.*, 2016; Pjevac *et*
186 *al.*, 2017). However, their abundance in wastewater treatment plants is lower than AOB,
187 which is likely due to the competitive advantage of AOB in systems with high substrate
188 concentrations (Costa *et al.*, 2006; Kits *et al.*, 2017). In general, further research is needed
189 to understand the role of comammox in the nitrification process in full scale WWTPs.

190 **Anaerobic ammonia oxidizing bacteria**

191 Anaerobic ammonia oxidation (anammox) is performed by anaerobic ammonia oxidizing
192 bacteria (AnAOB). Anammox is catalyzed by the sequential activity of the enzymes NIR,
193 hydrazine synthase (HZS) and hydrazine dehydrogenase (HDH) (Kartal *et al.*, 2013). All
194 three enzymes are located in the anammoxosome, an intra-riboplasmic compartment
195 (Niftrik *et al.*, 2004; Kartal *et al.*, 2011; Bagchi *et al.*, 2016; Bhattacharjee *et al.*, 2017). The
196 lumen of the anammoxosome is separated by three single bilayer membranes from the
197 environment (van Niftrik *et al.*, 2008). Additionally, the high density of the anammoxosome
198 membrane reduces passive proton diffusion (Sinninghe Damsté *et al.*, 2002, 2005). The
199 pH of the anammoxosome and the riboplasm of anammox cells is slightly acidic (pH 6.3)

and close to neutral (pH 7.3), respectively (van der Star *et al.*, 2010). AnAOB seem to be able to maintain the pH in their compartments up to ambient pH values of 7.8. Above pH 8.4 inhibition was observed, likely due to the collapse of proton motive force for ATP synthesis and a decrease in enzymatic activity (van der Star *et al.*, 2010; Puyol *et al.*, 2014). An ambient pH of 7.2-7.6 has been suggested as the optimum for AnAOB activity (Puyol *et al.*, 2014).

Heterotrophic denitrifying bacteria

Denitrification is a stepwise reduction pathway with several free intermediates ($2NO_3^- \rightarrow 2NO_2^- \rightarrow 2NO \rightarrow N_2O \rightarrow N_2$), and only when the pathway proceeds all the way to N_2 does it meet the strict definition of denitrification (Zumft, 1997). The four reductive steps are catalyzed by nitrate reductase (NAR), NIR, NOR and nitrous oxide reductase (NOS), respectively. This respiratory process is primarily carried out by heterotrophic denitrifiers (HD) under anoxic or suboxic conditions (Zumft, 1997), which are widely distributed in both soils and aquatic habitats (Knowles, 1982). HD in WWTPs are often closely affiliated with Proteobacteria and Bacteroidetes (Lu *et al.*, 2014). Denitrification rates are commonly measured as NO_3^- reduction rates and have been reported to increase with increasing pH with an optimum in the range of pH 7.0 to 8.0 (Van Cleemput and Patrick, 1974; Knowles, 1982). Recently, Šimek and coworkers suggested that the expression 'optimum pH for denitrification' should be avoided, as the results for pH optima obtained by different experimental methodology and denitrifier communities were incongruent (Šimek and Cooper, 2002; Šimek *et al.*, 2002). Studies have shown, that denitrification in activated sludge from WWTPs decreased at low pH values (pH < 6-6.5), generating N_2O as the major product, which suggests a non-uniform effect of pH on the different enzymatic

reactions during denitrification (Hanaki *et al.*, 1992; Thomsen *et al.*, 1994; Thörn and Sörensson, 1996). Recently discovered obligate N_2O reducers, that only possess the genetic potential to reduce N_2O to N_2, could be of interest in terms of N_2O mitigation strategies (Jones *et al.*, 2013; Graf *et al.*, 2014). Managing their activity in microbial communities could reduce net-N_2O production by increasing N_2O consumption.

Denitrifying anaerobic methane oxidizing bacteria and other bacteria hosting nitric oxide dismutase

A novel N conversion pathway has recently been discovered, in which microbes reduce NO_2^- to NO and then dismutate the NO to N_2 (and O_2) in a single enzymatic conversion step catalyzed by nitric oxide dismutase (NOD). This process is usually coupled with alkane oxidation by a monooxygenase (Ettwig *et al.*, 2012). Bacteria that harbor NOD in WWTPs are diverse and are especially abundant in systems with transient occurrence of NO_2^-/NO_3^- or hypoxia (Zhu *et al.*, 2017). They also have been detected in activated sludge systems and anaerobic digesters from municipal WWTP (Ho et al., 2013).Here we focus on *Methylomirabilis oxyfera* and related denitrifying anaerobic methane oxidizing bacteria (DAMO), which host NOD. *M. oxyfera* and related DAMO bacteria have been used for simultaneous nitrite and methane removal from effluents of anaerobic digestion processes (Cai *et al.*, 2015; López et al., 2016). Other relevant bacteria and archaea exist in DAMO driven systems, but they do not host NOD and are therefore not subject of discussion here.

NOD produces oxygen and thus exerts a control of the denitrifying pathway of DAMO. Once oxygen concentrations increase, the expression of NIR – that supplies NOD with its substrate NO – is downregulated, which results in a decrease of denitrification activity

(Luesken *et al.*, 2012). However, the enzyme that is responsible for aerobic methane oxidation (pMMO) in DAMO bacteria can scavenge the oxygen produced by NOD, thus avoiding the downregulation of NIR. pMMO is located in the cell membrane and is in contact with the periplasm (Wu *et al.*, 2011). The balance between pMMO and NOD enables DAMO bacteria to simultaneously oxidize methane aerobically and to denitrify NO_2^- anaerobically via NOD.

Little is known about a potential effect of pH on NOD and NOD hosting bacteria. Generally, further research is needed to identify the microbes that perform nitric oxide dismutation with NOD and to understand a potential impact of pH on them. Learning how to engineer systems that rely on NO dismutation could potentially decrease N_2O emissions from water treatment systems.

In summary, a wide range of growth pH optima have been reported for different microorganisms involved in the N-network (Fig. 3). Based on these optima, it is not feasible to deduce favorable pH set-points for wastewater treatment processes, as pH alone is likely insufficient to engineer the microbial community and select for or against functional microbial guilds to an extent that would reduce net-N_2O production in these systems. Instead, pH may be a suitable tool to manage fluxes through different enzymatic conversion routes, when a pH control at the enzymatic level proves successful.

3. The effect of pH on individual enzymes involved in N-conversion

Outside the pH optimum of an enzyme, a reduction in catalytic activity is mainly caused by changes in the enzyme's structural integrity (Illanes *et al.*, 2008). Changes of structure are caused by the disruption of covalent and non-covalent bonds in the polymers.

Furthermore, changes of the ionization state of amino acid residues in the active site of an enzyme can affect catalytic activity (Illanes *et al.*, 2008). Information about the ionization state of amino acid residues is relevant to understand how the activity of an enzyme may be affected by pH (Nelson and Cox, 2005). The isoelectric point (pI) defines the pH at which the occurrence of an amino acid in its protonated and de-protonated form is balanced; the pI is used in this review to inform the reader about the ionization states of amino acids. Amino acid residues stabilize metal ions and solvents, cause the secondary, tertiary and quaternary structure of an enzyme and form electron and proton transport routes within enzymatic complexes (Nelson and Cox, 2005).

The molecular structure has been solved for a reference of most of the enzymes discussed in this article (Table 1). The high-resolution structures reveal catalytically active sites, intramolecular electron/proton transfer pathways and electron acceptor/donor binding sites. All enzymes involved in the discussed N-conversion processes possess at least one of the following inorganic co-factors: iron-sulfur clusters (Fe-S), Molybdenum (as part of a molybdopterin cofactor) or Cu or Fe (often in the form of heme groups), all of which play relevant functional roles in the enzymes. The effect of pH on each of these structures appears to be consistent across the enzymes.

285 **Table 1 – Enzymes involved in N-transformation reactions.**

Enzyme	Full name	Reaction	pH optimum	Cellular location	Structure available	Catalytic atom
AMO	ammonia monooxygenase	$NH_3 + O_2 + 2H^+ + 2e^-$ -> $NH_2OH + H_2O$	Not reported (7.5 for related particulate methane monooxygenase (pMMO)) (Prior and Dalton, 1985; Zahn and DiSpirito, 1996)	Cytoplasmic membrane (Fiencke and Bock, 2006; Simon and Klotz, 2013)	Not available, refer to the structure of pMMO (Lieberman and Rosenzweig, 2005)	Likely Cu
HAO	hydroxylamine dehydrogenase	$NH_2OH + H_2O$ -> $HNO_2 + 4H^+ + 4e^-$ or $2NH_2OH + 1.5O_2$ -> $2NO + 3H_2O$	8.5 (Hooper *et al.*, 1984)	Periplasm (Igarashi *et al.*, 1997)	Yes (Igarashi *et al.*, 1997)	Fe (heme)
NXR *Nitrobacter*	nitrite oxidoreductase	$NO_2^- + 0.5O_2$ -> NO_3^-	Not reported	Cytoplasmic membrane (Cobley, 1976)	No	Mo
NXR *Nitrospira*	nitrite oxidoreductase	$NO_2^- + 0.5O_2$ -> NO_3^-	Not reported	Periplasm (Koch *et al.*, 2015)	No	Mo
NAR	nitrate reductase	$NO_3^- + 2H^+ + 2e^-$ -> $NO_2^- + H_2O$	7 ± 0.5 (Carlson *et al.*, 1982)	Cytoplasm (Berks *et al.*, 1995; Bertero *et al.*, 2003)	Yes (Moura *et al.*, 2004)	Mo
NAP	nitrate reductase	$NO_3^- + 2H^+ + 2e^-$ -> $NO_2^- + H_2O$	8 (Radcliffe and Nicholas, 1970); 6.5-7.5 (Carlson *et al.*, 1982)	Periplasm (Tavares *et al.*, 2006)	Yes (Jepson *et al.*, 2007)	Mo
Cu-NIR	nitrite reductase	$NO_2^- + 2H^+ + e^-$ -> $NO + H_2O$	<6.5 (Kim *et al.*, 2009); 5.6-6 (Wijma *et al.*, 2006)	Periplasm (Silvestrini *et al.*, 1994)	Yes (Fukuda *et al.*, 2016)	Cu
cd-NIR	nitrite reductase	$NO_2^- + 2H^+ + e^-$ -> $NO + H_2O$	5.8 (Richter *et al.*, 2002); 6.7 (Lam and Nicholas, 1969)	Periplasm (Silvestrini *et al.*, 1994)	Yes (Farver *et al.*, 2009)	Fe (heme)
cNOR	nitric oxide reductase	$2NO + 2H^+ + 2e^-$ -> $N_2O + H_2O$	6 (Field *et al.*, 2002); 5 (Flock *et al.*, 2005)	Cytoplasmic membrane (Hino *et al.*, 2010)	Yes (Hino *et al.*, 2010)	Fe
NOS	nitrous oxide reductase	$N_2O + 2e^- + 2H^+$ -> $N_2 + H_2O$	7 (Fujita and Dooley, 2007); 8 (Johnston *et al.*, 2017)	Periplasm (Pauleta *et al.*, 2013)	Yes (Pauleta *et al.*, 2013)	Cu
HZS	hydrazine synthase	$NH_4^+ + NO + 2H^+ + 3e^-$ -> $N_2H_4 + H_2O$	Not reported	Anammoxosome (Kartal *et al.*, 2011)	Yes (Dietl *et al.*, 2015)	Fe (heme)
HDH	hydrazine dehydrogenase	N_2H_4 -> $N_2 + 4H^+ + 4e^-$	8 (Schalk *et al.*, 2000); 8-8.5 (Shimamura *et al.*, 2008)	Anammoxosome (Kartal *et al.*, 2011)	Yes (Maalcke *et al.*, 2016)	Fe (heme)

NOD	nitric oxide dismutase	2NO -> N_2 + O_2	Not reported	Periplasm (Wu *et al.*, 2011)	Partly (in comparison to qNOR) (Ettwig *et al.*, 2012)	Fe (heme)

3.1. The effect of pH on catalytic metal atoms

All enzymes discussed in this article contain metal ions as co-factors (Table 1). Variations of pH alter the protonation states of amino acid residues in proximity of these co-factors and affect the steric organization of the metal ions, which in turn affects the catalytic activity of the enzymes (Nelson and Cox, 2005).

Copper

Copper atoms are part of the catalytic sites of Cu-Nitrite reductase (Cu-NIR) and nitrous oxide reductase (NOS) (Table 1). Cu-atoms are likely also part of the catalytic site of ammonia monooxygenase (AMO), although a molecular structure of the enzyme has so far not been obtained (Zahn and DiSpirito, 1996).

Cu-NIR contains two Cu-sites per homotrimer, type-1 and type-2, each of which contains one Cu-atom (Jacobson *et al.*, 2007). The type-1 copper site is located close to the surface of Cu-NIR and accepts an electron from electron donors like pseudoazurin (Kukimoto *et al.*, 1996). The electron from the type-1 copper site is transferred to the type-2 copper in the active site to reduce a NO_2^- molecule (Suzuki, 1994). The type-2 copper site is located between two monomers of Cu-NIR and is directly involved in catalysis – NO_2^- binds to the copper ion (Godden *et al.*, 1991). High pH (pH 8.4) leads to the deprotonation of amino acid residues in the proximity of the type-2 Cu-ion and also results in a loss of protons of the solvent ligand in proximity of the type 2 Cu-ion (Jacobson *et al.*, 2007). The solvent ligand has been suggested, depending on the pH, to be either a water

molecule or hydroxide ion that contributes with the presence or absence of its proton to the active site geometry. With the absence of protons from amino acid residues and the solvent ligand at pH 8.4 structural transformations of the type-2 copper site geometry were reported, which change the site geometry from a nearly regular tetrahedron into a more distorted tetrahedral geometry (Godden *et al.*, 1991; Jacobson *et al.*, 2007). It was hypothesized that the type-2 copper site is unable to accept an electron from the type-1 copper site in the distorted geometry, which may explain the rapid decrease of enzyme activity with increasing pH (measured in the range pH 6.0 to 9.0)(Jacobson *et al.*, 2007).

NOS also contains two Cu-centers, CuA and CuZ (Pauleta *et al.*, 2013; Carreira *et al.*, 2017). CuA is located close to the surface of the enzyme and accepts electrons from the physiological electron donors cytochrome c_{550} and pseudoazurin, before passing them on to CuZ (Mattila and Haltia, 2005; Gorelsky *et al.*, 2006). CuZ constitutes the catalytic site and contains four Cu-atoms, which are bridged by two sulfur atoms (Pauleta *et al.*, 2013). The intramolecular electron transfer between CuA and CuZ appears to be rate limiting for N_2O reduction in the range of 4<pH<8, when the electron transfer rate to CuZ is hampered due to conformational changes of CuA (Gorelsky *et al.*, 2006). However, at more alkaline pH (pH > 8.9) a loss of catalytic activity was also observed, likely due to the deprotonation of a lysine residue (pI = 9.7) in CuZ, which in its deprotonated form cannot stabilize the Cu_Z° intermediate anymore (Johnston *et al.*, 2017). Therefore, the protonation of the lys397 residue appears to be essential for a successful reduction of N_2O via Cu_Z°. The balance between both effects of pH, either the effect of pH on the electron transfer from CuA to CuZ or the protonation state of lys397, may contribute to an observed pH optimum of pH 7-8 (Fujita and Dooley, 2007; Johnston *et al.*, 2017).

Iron

Iron atoms play a central role in almost all enzymes involved in the N-network and act either as catalysts in the active sites or mediate electron transfer within an enzymatic complex. The iron atoms in the active sites are often (e.g. HAO, cd-NIR, HZS, HDH, cNOR), but not always (e.g. cNOR; cNOR contains one heme and one non-heme Fe) part of heme groups. The Fe-atoms in heme groups commonly interact with proximal histidine (his) residues (pI=7.6) (Igarashi *et al.*, 1997; Maalcke *et al.*, 2016). Protonated his-residues move the Fe-atom out of the heme plane and cause the Fe-atom to change its spin state (Perutz *et al.*, 1998). The switch of spin state R to T results in a change of free energy that eventually results in a decrease of substrate affinity and catalytic rates (details in (Perutz *et al.*, 1998)). In order to successfully stabilize heme Fe-atoms, his-residues apparently need to be present in their deprotonated form. However, the role of his-residues is not limited to the stabilization of Fe-atoms. For instance, in cd-NIR his-residues are directly involved in the binding of substrate, when a his-residue binds one of the oxygen atoms of the substrate NO_2^- by hydrogen bonds (Tavares *et al.*, 2006). cd-NIR is highly reactive *in vitro* at pH<7, but poorly active at alkaline pH, when his-residues are deprotonated (Tavares *et al.*, 2006).

Molybdenum

The molybdenum containing molybdo-bis (pyranopterin guanine dinucleotide) co-factor is characteristic for NXR, NAR and NAP and catalyzes either oxidative hydroxylation or reductive dehydroxylation, in which the hydroxyl group is derived from water (Rajagopalan and Johnson, 1992). In principle, one Mo-atom is coordinated with two pyranopterins and interacts with cys- (NAP) or ser- or asp-residues (NAR)(pI=5.1, 5.7 and 2.8, respectively)(Moura *et al.*, 2004). Two conserved his-residues in the proximity of the

pyranopterin rings appear to be of significance for the catalytic activity of NAR (Bertero *et al.*, 2003; Wu *et al.*, 2015). In their protonated form the his-residues stabilize the pyranopterin rings and the Mo(V) intermediate during catalysis. Upon loss of an overall positive charge, the midterm potential of the molybdenum ion decreases, which is directly correlated with enzyme activity (Wu *et al.*, 2015). Therefore, an increase of pH appears to directly transfer into a loss of catalytic turnover of NAR.

For NAP the exact mechanism of how pH affects the catalytic activity is unknown. The ionization state of thiol ligands appears to be relevant for the activity of the Mo-ion (Axley *et al.*, 1991; Jepson *et al.*, 2007) and similar causalities as for NAR may apply. However, it needs to be verified, if the findings on the effect of pH on NAR are also valid for NAP.

3.2. The effect of pH on intramolecular proton and electron transport

The effect of pH on enzymes is not limited to amino acid residues in proximity of catalytic metal ions. The transfer of protons and electrons towards and away from the catalytic sites is also affected by pH. Proton transport usually occurs through channels in the three-dimensional structure and is mediated by initially deprotonated amino acid residues. For example, in cNOR the transfer of protons from the periplasm to the active site is crucial for NO reduction activity (Flock *et al.*, 2008). Protons are channeled by a series of glu-residues (pI=3.2) from the surface of the enzyme to the catalytic heme centers. The exact mechanism of proton transport is unknown, but the transfer is strongly affected by pH and decreases with increasing pH from k_{max} = 250 s^{-1} at pH 5 to k_{obs}=10 s^{-1} at pH 8 (Flock *et al.*, 2005). The transfer of protons likely becomes the rate limiting step of cNOR at neutral and alkaline pH (Flock *et al.*, 2005).

In HZS it has been proposed that a cluster of polar amino acid residues (γAsp112, pI=2.8; γArg143 and γArg167, pI=10.8) between γAsp168 and the surface mediates proton transfer to the active site (Dietl *et al.*, 2015). However, the exact mechanism is unknown. The effect of changing pH on the proton transfer of HZS has not been studied and may in fact be of low relevance, as the pH inside the anammoxosome has been reported to be stable (approx. pH 6.3)(van der Star *et al.*, 2010).

Electron transfer is usually mediated by series of heme or Fe-S clusters (HAO, NXR, NAR, NAP, cd-NIR, cNOR) that accept electrons from electron donors, like cytochromes, at the surface of the enzymatic complexes and transfer them sequentially to the active sites. The edge-to-edge distance between heme or Fe-S clusters has to be short enough for direct electron transfer, e.g. 15 Å in HZS or 7 Å and 11 Å in NAR (Bertero *et al.*, 2003; Dietl *et al.*, 2015). Larger distances can be bridged by amino acid residues (Bertero *et al.*, 2003; Dietl *et al.*, 2015). For instance, in HZS it has been proposed that three electrons from the redox partner kuste2854 enter the enzymatic complex via a haem (γII) close to the surface of HZS (Dietl *et al.*, 2015). The electrons may then be transferred to another haem (γI) that is situated in the active site. A his-residue (γHis144) that is located between γI and γII may play an important role in the electron transfer between both haems and its protonation state may affect electron transfer rates. However, in general, it is not well understood how pH affects these electron transport paths in detail, although effects of pH have been described in some cases: In NAR for example the electron transfer to the Mo-center occurs via [Fe-S] clusters and is affected by pH (Bertero *et al.*, 2003; Rothery *et al.*, 2004). The protonation state of Arg-residues (pI=10.8) may play a role in altering the midterm potential of the [Fe-S] clusters, but the exact mechanism is unknown (Bertero *et al.*, 2003).

In summary, molecular structures with their detailed information about the position of amino acid residues, ligands and metal atoms give valuable insights in how variations in pH cause changes in catalytic sites, ligand affinity or electron and proton transport routes. The insights on the molecular level may also facilitate the interpretation of changes of overall enzyme kinetics with pH. However, findings on the molecular level have to be augmented with kinetic data in order to quantify the effect of pH on enzymatic conversion rates.

3.3. pH optima of enzyme activity

3.3.1. The discrepancy between *in vitro* and *in vivo* enzyme kinetics.

pH optima of enzyme activity are commonly obtained by *in vitro* activity assays, when purified enzymes, substrates and often non-physiological activation agents and electron acceptors are introduced to an assay medium. Usually, the conditions applied are optimized for good signal-to-noise ratios and differ from conditions in the cellular environment (García-Contreras *et al.*, 2012). Also, when kinetics of different enzymes, that interact in the cell in the same environment, are obtained under different assay conditions, uncertainty about the properties of enzymatic cascades is introduced. In recent years attempts have been made to obtain enzyme kinetics that represent *in vivo* conditions by setting with physiologically relevant assay conditions (van Eunen and Bakker, 2014), including the buffer capacity and anion composition, macromolecular crowding and pH. However, most pH optima listed in databases to date were not obtained by *in vivo*-like assays. Accordingly, their validity for physiological conditions needs to be handled with care. Until *in vivo*-like enzyme kinetics are more widely reported, pH optima of enzyme

activity from *in vitro*-assays may serve as a starting point for considerations about favorable pH set-points.

3.3.2. pH optima of NIR, NOR and NOS in respect to N_2O net-production

When studying the pH optima of the enzymes that are metabolically up- and downstream of N_2O in the N-network, the enzymes NIR and NOR and the enzyme NOS with acidic and alkaline pH optima, respectively, stand out (Fig. 4). The difference suggests that pH may regulate the rates between N_2O production and consumption.

Figure 4

NOR and NIR play an important role in N_2O production, as they either produce N_2O directly (NOR) or produce NO (NIR), a precursor of N_2O. NO is a free radical with cytostatic or –toxic properties. In order to keep intracellular NO concentrations low, microorganisms have developed rapid detoxification mechanisms that involve reduction of NO to N_2O, e.g. by NOR or $cytP_{460}$ (Stein, 2011; Caranto *et al.*, 2016). Oxidation rate constants for NOR are pH dependent and decrease with increasing pH (oxidation rate constants of NOR at pH 6.0 = 100 s^{-1} and at pH 7.5 = 12 s^{-1} (Lachmann *et al.*, 2010)). Therefore, N_2O production by NOR decreases at alkaline pH.

NIR catalyzes the reduction of NO_2^- to NO, a reaction that is reversible. In fact, it has been reported that the directionality of Cu-NIR of *Alcaligenes faecalis* is strongly dependent on pH (Wijma *et al.*, 2004). K_{cat} for nitrite reduction was twelve times higher at pH 6.0, than at pH 8.0. The catalytic bias of NIR towards oxidation of NO to NO_2^- at pH 8, rather than reduction of NO_2^-, is caused by an altered reduction potential of the electron donor pseudoazurin and hydroxyl inhibition above pH 7 (Wijma *et al.*, 2004). However, as the

reduction of NO_2^- to NO clearly occurs during denitrification at pH 7-8 in natural systems, the effect of pH on the enzyme is unlikely the only factor that determines the catalytic directionality of NIR. The findings are particularly interesting in the context of the recently proposed role of Cu-NIR during nitritation (Caranto and Lancaster, 2017). Further research will inform if the bidirectionality of Cu-NIR determines the fate of NO during nitrification and denitrification and thus acts as a source or sink of NO as a precursor of N_2O.

In contrast to NIR and NOR, the activity of NOS determines, whether N_2O or N_2 is the final product of denitrification (as comprehensively reviewed in (Carreira *et al.*, 2017)). High activity of NOS increases N_2O consumption and has the potential to decrease net N_2O production. The N_2O:N_2 product ratio of denitrification increases with decreasing pH and conversely decreases with increasing pH (Šimek and Cooper, 2002; Bergaust *et al.*, 2010) . However, an increase of N_2O:N_2 at acidic pH is likely not caused by lower gene copy numbers of *nosZ*, nor lower transcription rates of nosZ vs. nirS that may explain an imbalance between N_2O production and consumption rates; on the contrary, the ratio of *nosZ/nirS* transcripts is larger at lower pH (pH = 6.1), than at higher pH (pH = 8.0)(Liu *et al.*, 2010). pH seems to affect the N_2O:N_2 ratio mainly on the post-transcriptional level, when higher pH determines the successful assembly of functional NOS at pH >7, whereas at pH ≤6.1 the assembly of functional NOS seems to be disturbed (Liu *et al.*, 2014).

Does the effect of pH on NIR, NOR and NOS mean that for lower N_2O net-production, N-removal processes should simply be operated at more alkaline pH to promote N_2O consumption over production? A direct correlation between pH optima of individual enzymes and the N_2O net-production of entire N-removal processes is unlikely, as enzymes cannot be treated as independent entities. They depend on electrons and

465 substrates from other enzymatic conversion steps. Therefore, the comparison of individual
466 enzyme activities as a function of pH alone does not necessarily hold sufficient information
467 to evaluate the effect of pH on transformation rates of entire pathways.

468 4. The effect of pH on pathway conversion rates and net-N_2O 469 production

470 Here the effects of pH on the pathway level are reviewed (Fig. 5). Five pathways are
471 considered: a) Nitritation, b) Nitrifier denitrification, c) Anaerobic ammonia oxidation, d)
472 Heterotrophic denitrification and e) Oxygenic denitrification of nitrite.

473 **Figure 5**

474 4.1. Nitritation

475 *Nitritation (AOB)*

476 Nitritation has been understood as the sequential oxidation of NH_3 to NO_2^- via NH_2OH, in
477 which the oxidation of NH_3 to NO_2^- is catalyzed by AMO and HAO (Kostera *et al.*, 2010).
478 Recently, *in vitro* experiments showed that HAO reduces NH_2OH in a three electron
479 oxidation to NO, instead of a four electron oxidation to NO_2^- (Caranto and Lancaster,
480 2017). The occurrence of NO_2^-, as observed in previous NH_2OH oxidation experiments,
481 would then either be attributed to a subsequent non-enzymatic oxidation of NO to NO_2^- by
482 O_2 or an enzymatic oxidation step (potentially via Cu-NIR). An enzymatic oxidation of NO
483 to NO_2^- would preserve the fourth electron from NH_2OH oxidation in the electron pool to
484 contribute to the generation of the proton motive force (Caranto and Lancaster, 2017).

485 From a thermodynamic perspective, nitritation is favored at alkaline pH, when the Gibbs
486 free energy for NH_3 oxidation is larger at neutral and alkaline pH, than at acidic pH

($\Delta G^{\circ}_{25°C,pH\ 7}$ = -274.7 kJ/kmol N vs. $\Delta G^{\circ}_{25°C,pH\ 4}$ = -240.5 kJ/kmol N)(Wrage *et al.*, 2001). pH also influences the bioavailability of cytochromes as reaction partners of e.g. HAO and thus may affect nitritation rates. At pH 10 cytochrome c_{554} was shown to be three times more available in the periplasm in its required soluble form, than at pH 8, when it was adhered to the membrane (McTavish *et al.*, 1995). Nitritation rates may therefore not only be limited by the activity of enzymes, but also the availability of downstream electron carriers.

The current model of electron flow in AOB during nitritation describes that the four electrons released by HAO are transferred to electron acceptors, either cytochrome 554 (c554) or directly to membrane bound c_m552. The electrons then enter the ubiquinone pool possibly via a designated "Hydroxylamine Ubiquinone Redox Module" (HURM) (Hooper *et al.*, 1997; Simon and Klotz, 2013; Perez-Garcia *et al.*, 2014). Two electrons are channeled back to AMO for the reduction of O_2, while the remaining electrons are used to establish the proton motive force. However, the exact pathways of electron transport in AOB during nitritation remain to be resolved and may involve other redox mediators, such as c552. Additionally, direct interactions between the cytochromes that alter the electron flow cannot be excluded, e.g. c554 with c_M552 (Simon and Klotz, 2013; Stein *et al.*, 2013; Kozlowski *et al.*, 2016). The model needs to be refined in respect to the findings of NH_2OH oxidation to NO (Caranto and Lancaster, 2017). However, the proposed three electron oxidation of NH_2OH to NO by HAO may be compatible with the current model. The flow of one electron to NirK for the reduction of NO_2^- to NO during denitrification, which is commonly mediated by cytrochromes $c_{550\text{-}553}$ or (pseudo) azurins or cupredoxins (Simon and Klotz, 2013), may be reversed and that way contribute an electron to the generation of the proton motive force. It has been demonstrated that the reduction of NO_2^- to NO is

511 reversible and that the catalytic equilibrium is dependent on pH: at acidic pH (pH 6.2) NO_2^-
512 reduction occurred at faster rates, than NO oxidation, while at pH 8.0 NO oxidation to NO_2^-
513 was faster (Wijma *et al.*, 2004). Therefore, the pH may be relevant for the accumulation of
514 NO during nitritation.

515 In the proposed nitritation pathway by Caranto and Lancester, i.e. NH_3 -> NH_2OH -> NO ->
516 NO_2^-, the balance between HAO and NO oxidation rates, potentially by NirK, are relevant:
517 a low oxidation rate of NirK would result in the accumulation of NO and potentially to
518 increased N_2O production due to reduction of accumulated NO to N_2O. Both HAO and
519 NirK are located in the periplasm and are affected by ambient pH. The pH optima of both
520 enzymes are substantially different (pH 8.5 for HAO vs. 6.0 for NirK). Lower pH would
521 result in relatively higher NO oxidation rates by NirK, compared to NH_2OH oxidation rates
522 of HAO, which would prevent accumulation of NO. In contrast, at alkaline pH NO oxidation
523 rates of NirK may be lower, than NH_2OH oxidation rates by HAO, which may lead to
524 accumulation of NO and potentially increased N_2O production rates.

525 Observations from nitrifying AOB enrichment cultures and cultures of *N. europaea* support
526 the stimulating effect of pH on nitritation and N_2O production rates (Shammas, 1986). pH
527 optima and minima for N_2O production rates were identified at pH 8.0-8.5 and below pH
528 7.0, respectively (Hynes and Knowles, 1984; Law *et al.*, 2011; Rathnayake *et al.*, 2015). In
529 summary, alkaline pH appears to favor higher nitritation rates and higher N_2O production
530 rates by AOB. The role of the different enzymes involved in nitritation and potential
531 imbalances of metabolic fluxes between them will be elucidated in future studies.

532 *Nitritation (AOA)*

N_2O production during ammonia oxidation has been detected in pure and enrichment cultures of AOA (Jung *et al.*, 2014; Stieglmeier *et al.*, 2014). Compared to the better elucidated N_2O production pathways of AOB, the mechanisms of N_2O production by AOA are not well understood, due to the lack of physiological and genomic evidence for enzymatic processes for N_2O production (Walker *et al.*, 2010; Tourna *et al.*, 2011; Spang *et al.*, 2012). Recently, NO and NH_2OH have been identified as essential intermediates during archaeal ammonia oxidation, however both intermediates are probably reduced to N_2O non-enzymatically (Vajrala *et al.*, 2013; Zhu-Barker *et al.*, 2015; Kozlowski *et al.*, 2016). This contrasts to the enzymatic N_2O production in bacteria (Walker *et al.*, 2010; Tourna *et al.*, 2011; Spang *et al.*, 2012; Stieglmeier *et al.*, 2014). Hence, the production of N_2O during ammonia oxidation in soil was suggested to be lower for AOA, than AOB (Hink *et al.*, 2017). pH selects for different bacterial and archaeal communities and therefore determines the relative contribution of AOA and AOB to overall N_2O production (Nicol *et al.*, 2008). In fact, AOA become the dominant contributors to nitritation and respective N_2O production rates in low pH environments, e.g. soils (Li *et al.*, 2018). The production of N_2O by AOA in wastewater treatment systems has, to our knowledge, not been documented as relevant.

4.2. Nitrifier denitrification

Nitrifier denitrification (ND) describes the reduction of NO_2^- to N_2O by NIR and NOR in AOB (Wrage *et al.*, 2001). Rates of ND increase at low oxygen tension. In the current model a bottleneck of electron flow occurs at low oxygen tension between HAO and the terminal oxidase, likely between HAO and c_M552 or in the c552 pool between the cytochrome complexes C-III and C-IV, as a result of insufficient availability of oxygen as an electron acceptor (Simon and Klotz, 2013; Kozlowski *et al.*, 2016). In order to relieve the

557 bottleneck NIR and NOR may be activated and accept electrons from c552 to reduce NO_2^-
558 and NO to N_2O, thus constituting the nitrifier denitrification pathway (Kozlowski *et al.*,
559 2016). However, the regulation of electron flow in AOB does not follow a single principle
560 and different phylotypes appear to apply different regulatory features, which likely depend
561 on the genomic history and environment (Stein *et al.*, 2013).

562 A relevant role of regulation may be assigned to the small red-copper protein
563 nitrosocyanin. Nitrosocyanin may regulate whether electrons from HAO are transferred to
564 c554 or c_M552, thus determining their fate in the electron flow cascade of AOB (Stein *et*
565 *al.*, 2013). Electrons loaded to c_M552 would directly enter the quinone pool to be
566 distributed to AMO, whereas electrons loaded to c554 would be available for further
567 distribution to other electron carriers, e.g. c552, and to NIR and NOR. Therefore,
568 nitrosocyanin may act as a regulator of electron flow in order to obtain redox balance in
569 environments with sufficiently high substrate concentrations (Stein *et al.*, 2013).

570 It is challenging to study the rate of nitrifier denitrification isolated. Accordingly, a pH
571 optimum is difficult to obtain. Based on the pH optima of NIR and NOR, it may be
572 hypothesized that elevated rates of ND occur at slightly acidic pH (Table 1). However, as
573 mentioned above, ND occurs in a complex interaction with the cellular electron pool and
574 different electron carriers and a deduction of a pH optimum only from NIR and NOR is
575 likely too simple. It may be speculated that, similar to c_{554} (4.1.,(McTavish *et al.*, 1995)), an
576 effect of pH also on c_{552} or nitrosocyanin may play a role for the conversion rates of NIR
577 and NOR. However, it is currently unknown, if the pH affects the concentration of c_{552} or
578 nitrosocyanin directly or indirectly or how pH affects nitrifier denitrification rates in general.

4.3. Anaerobic ammonia oxidation

The anaerobic ammonia oxidation (anammox) pathway comprises three sequential steps in which the substrates NH_4^+ and NO_2^- are converted into N_2 (Strous *et al.*, 2006; Kartal *et al.*, 2011, 2013). Initially, NO_2^- is reduced to NO by NIR. Then, NH_4^+ is oxidized with NO to hydrazine (N_2H_4) by HZS. Finally, N_2H_4 is oxidized to N_2 by HDH. All enzymes involved are located in the anammoxosome. The transfer of electrons and protons between the three enzymatic reactions has been proposed to be mediated by the quinol::cytochrome c oxidoreductase system (complex III, bc1 complex) in the membrane of the anammoxosome (Kartal *et al.*, 2011). The anammox pathway does not include N_2O as an intermediate (Strous *et al.*, 2006; Kartal *et al.*, 2013) and is generally not considered to yield N_2O. Although the enzymes involved in anammox are located in the anammoxosome, and therefore a change of ambient pH is unlikely to affect the enzymes directly, anammox rates increase with pH and are highest between pH 7.5-8.3. An explanation for this paradox may be found in an inhibitory effect of pH on other cellular processes, than the enzymatic conversion reactions themselves, which results in changes of anammox rates with pH. Substrate transporters have been proposed to play a significant role in substrate trafficking of AnAOB cells and an effect of pH on these proteins is likely, yet largely unknown (Kartal *et al.*, 2013).

4.4. Heterotrophic denitrification

Heterotrophic denitrification (HD) refers to the reduction of NO_3^- or NO_2^- to gaseous products (NO, N_2O and N_2), in which NO_3^- or NO_2^- serve as terminal electron acceptors. HD involves the enzymes NAR, NIR, NOR and NOS. HD comprises both N_2O production and consumption reactions. The ratio of complete (N_2) and incomplete (N_2O) denitrification

as a function of pH is relevant in respect to net-N_2O production. Various studies, especially from agricultural soils, report an increased $N_2O/(N_2+N_2O)$ ratio with decreasing soils (Šimek and Cooper, 2002; Bergaust *et al.*, 2010; Bakken *et al.*, 2012), which was assigned to an impaired post-transcriptional assembly of NOS at pH < 7.0 (Liu *et al.*, 2014). The effect of a relatively higher $N_2O/(N_2+N_2O)$ ratio at low pH may however be compensated by a decline in overall denitrification rates with decreasing pH (NO_3^- reduction rates increase with pH with an optimum between pH 7.0 and 8.0 (Van Cleemput and Patrick, 1974; Knowles, 1982)). Absolute N_2O production rates from denitrification can therefore be expected to be highest in the range pH 7.0-7.5, before N_2O reduction by NOS becomes effective at pH > 7.5 (Liu *et al.*, 2014).

4.5. Oxygenic denitrification of nitrite

The oxygenic denitrification of nitrite is a two steps process, in which nitrite is first reduced by cd-NIR to NO. NO is then further dismutated to N_2 and O_2 by NOD. The process bypasses N_2O as an intermediate towards N_2. An effect of pH on process rates of oxygenic denitrification of nitrite has, to our knowledge, not been reported yet. NIR and NOD are usually located in the periplasm and are likely exposed to changes of ambient pH. It may therefore be hypothesized that denitrification rates decrease, when pH diverges from the pH optimum of NIR (pH 5.8-6.7) and NOD (pH optimum not reported). The pH optimum of pMMO (pH 7.5; (Zahn and DiSpirito, 1996)) may also play a role, as reduced activity of pMMO may lead to accumulation of oxygen that in turn inhibits the denitrification process. However, with the current knowledge no reasonable pH optimum for oxygenic denitrification of nitrite via NOD can be identified.

In summary, slightly alkaline pH results in higher rates of nitritation, anammox and complete denitrification. Although changes of nitrifier denitrification and nitratation rates with pH have not been assessed in detail, rates of ND and nitratation are expected to decrease with increasing pH based on information available for the enzymes involved in these pathways (Fig. 5). The link between enzymatic pH optima and pathway conversion rates may enable predictions of net-N_2O production rates.

5. Exploring favorable pH set-points for lower net-N_2O production in wastewater bio-reactors.

Based on the findings of the previous sections, this last section hypothesizes favorable pH set-points (in respect to lower net-N_2O production rates) for nitrogen removing bio-reactors in wastewater treatment applications. The microbial communities in these systems commonly comprise AOB, AnAOB, NOB and HD. In respect to N_2O production AOB appear to be of most significance, whereas HD play a dominant role for N_2O consumption. As both functional guilds, i.e. AOB and HD, are present in essentially all nitrogen removing wastewater systems like nitrification-denitrification, partial nitrification or partial nitritation-anammox, considerations may be transferable between applications. The effect of pH on net N_2O production rates in these systems is hypothesized in three scenarios: pH 6.0-6.5, pH 7.0-7.5 and pH 8.0-9.0.

Slightly acidic pH 6.0-6.5

At slightly acidic pH, e.g. pH 6.5, nitritation rates are not at maximum (see 4.1), as the pH optimum of HAO lies at approx. 8.0-8.5. The proximity to the pH optimum of Cu-NIR (approx. pH 6, see Fig. 4) would result in an efficient removal of NO. Both N_2O production

and consumption rates are expected to be low at slightly acidic pH, which results in low net N_2O production rates. Therefore, lower net N_2O production rates can potentially be achieved in N-removing bioreactors by operation at slightly acidic pH, yet at the cost of reduced nitritation rates. This mode of operation may be interesting for nitrifying and partially nitrifying applications.

Neutral pH 7.0-7.5

At neutral pH, e.g. pH 7.0-7.5, nitritation rates increase, as reaction conditions are closer to the pH optimum of HAO. NH_2OH oxidation rates by HAO would be larger than NO oxidation rates by Cu-NIR and accumulated NO would be reduced to N_2O by either NOR or other detoxification mechanisms. N_2O production rates are therefore expected to be higher at pH 7.0-7.5, than at pH 6.5. Net N_2O production rates are also expected to be high, as the pH is not high enough to allow an efficient reduction of N_2O to N_2 by NOS. Out of all three pH scenarios net-N_2O production rates are expected to be highest in the range pH 7.0-7.5. This pH range appears as unfavorable for nitrogen removing applications, e.g. nitrification-denitrification, partial nitrification and partial nitrification-anammox, in respect to net N_2O production.

Alkaline pH 8.0-9.0

NH_2OH oxidation rates would be maximal at pH 8.0-9.0 and NO oxidation rates by Cu-NIR would be elevated. N_2O production rates are expected to peak at alkaline pH. However, other than at pH 7.0-7.5, high N_2O consumption rates may counterbalance production rates at alkaline pH, thus causing low net-N_2O production rates (see Fig 5). Therefore, alkaline pH of approx. 8.0 appears to be favorable for wastewater treatment applications, as net N_2O production rates may be low, while ammonia removal rates can be maintained.

669 In summary, the net N_2O production curve with pH would be “bell-shaped” with low net
670 N_2O production rates at pH ≤ 6.5, high rates at pH 7.0-7.5 and low rates at pH ≥ 8.0.

671 6. Conclusion

672 When N_2O production exceeds N_2O consumption microbial communities constitute
673 sources of N_2O. The microbial guilds that drive nitrogen conversion in wastewater
674 treatment processes (ammonia oxidizing bacteria or archaea, complete or anaerobic
675 ammonia oxidizing bacteria, nitrite oxidizing bacteria and heterotrophic denitrifying
676 bacteria) all have physiological growth optima in the range pH 7.0-8.0. Selecting specific
677 functional microbial guilds based on pH does, therefore, not appear feasible for the
678 mitigation of net N_2O production in complex microbial communities.
679 All enzymes that are of particular relevance for N_2O production and consumption are either
680 located in (i.e. HAO, NIR and NOS) or in contact with the periplasm, when embedded in
681 the cytoplasmic membrane (i.e. NOR). Accordingly, they are exposed to changes in
682 ambient pH. In general, pH alters the catalytic activities of enzymes involved in nitrogen
683 conversion, mainly by affecting their structural integrity. However, its effects vary: in Cu-
684 NIR and NOR, protonation or deprotonation of amino acid residues alter the steric
685 organization of catalytic sites (Cu-NIR, cNOR) or the substrate affinity of catalytic metal
686 ions (Cu-NIR, cd-NIR), which leads to sub-optimal conversion rates at pH > 7.0. Electron
687 and proton transfer within enzymatic complexes is also affected by pH. In HZS and NAR
688 electron transport becomes rate limiting with increasing pH, and hampered proton
689 transport reduces the turnover of cNOR 25-fold from pH 5 to 8. Finally, pH also exerts a
690 post-translational effect and prevents the assembly of NOS to a functional enzyme at pH <
691 7.0. The result is a decrease of N_2O consumption rates at acidic pH.

The different effects of pH on the molecular level are consistent with rate measurements of different bacterial N-conversion reactions, e.g. nitritation and denitrification, or the N_2O/N_2 product ratio of denitrification. Nitritation and N_2O production rates by AOB increase with pH in the range pH 6.0-8.0, while N_2O consumption by heterotrophic denitrifiers increases with pH > 7.0. Overall, net N_2O production in bacterial communities of wastewater engineering applications at low pH < 7 appears to be dominated by N_2O production, whereas at pH > 7.5 N_2O consumption counteracts production and leads to reduced net N_2O production rates. Therefore, lower net N_2O production rates can be achieved at acidic pH < 7.0, however at the cost of lower NH_4^+ removal rates. Low net N_2O production at simultaneously high NH_4^+ removal rates is possible at alkaline pH > 7.5 due to N_2O consumption in microbial communities that host denitrifying bacteria. Highest net N_2O production rates would occur at pH 7.0-7.5, when N_2O consumption does not outweigh increased production.

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Originality-Significance Statement

The presented work contributes to research in environmental microbiology by identifying the effects of pH on the enzymatic level of nitrogen conversion reactions, by identifying trends of conversion rates and relative N_2O production of relevant pathways upon changes

of pH, by connecting the effects of pH on the enzyme level with resulting changes on the pathway level and by hypothesizing favorable pH set-points for wastewater treatment applications with the scope of lower net-N_2O production.

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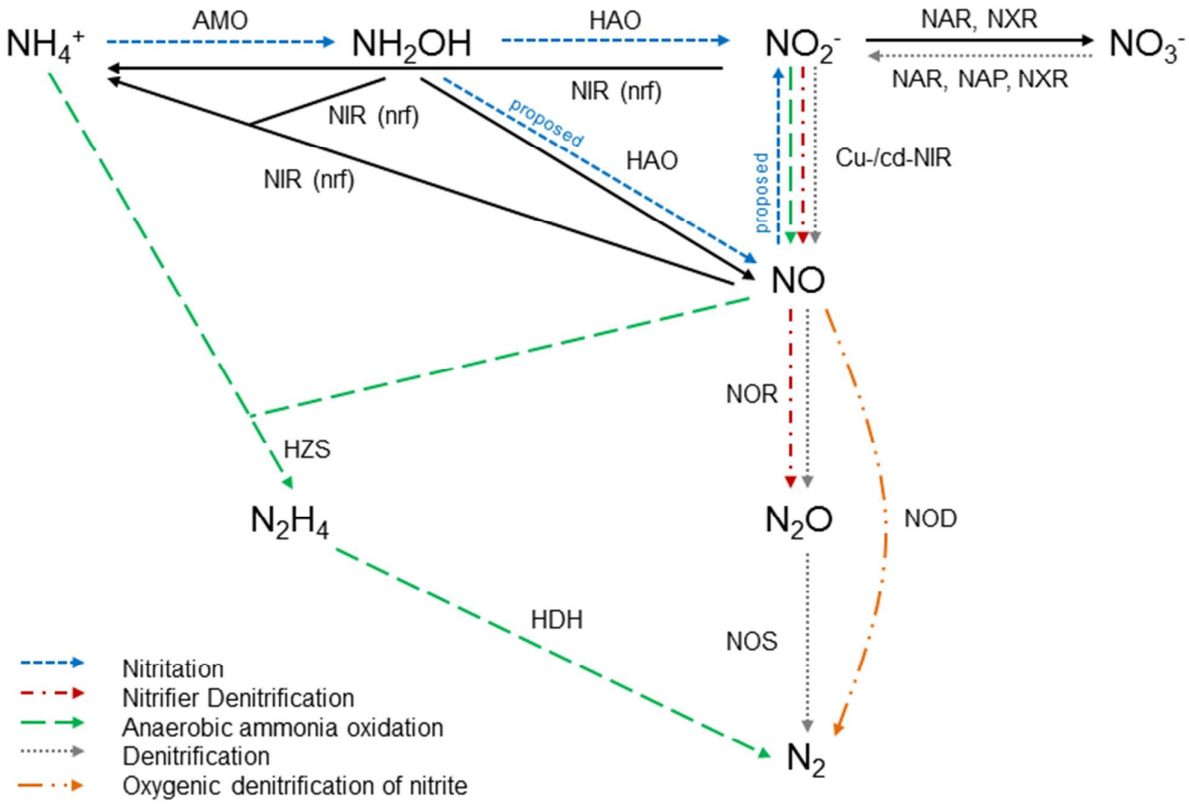

Figure 1 - Web of nitrogen conversion reactions. Enzymes involved in catalysis are ammonia monooxygenase (AMO), hydroxylamine dehydrogenase (HAO), nitrate reductase (NAR), periplasmic nitrate reductase (NAP), nitrite oxidoreductase (NXR), nitrite reductase (NIR), nitric oxide reductase (NOR), nitrous oxide reductase (NOS), hydrazine synthase (HZS), hydrazine dehydrogenase (HDH) and nitric oxide dismutase (NOD).

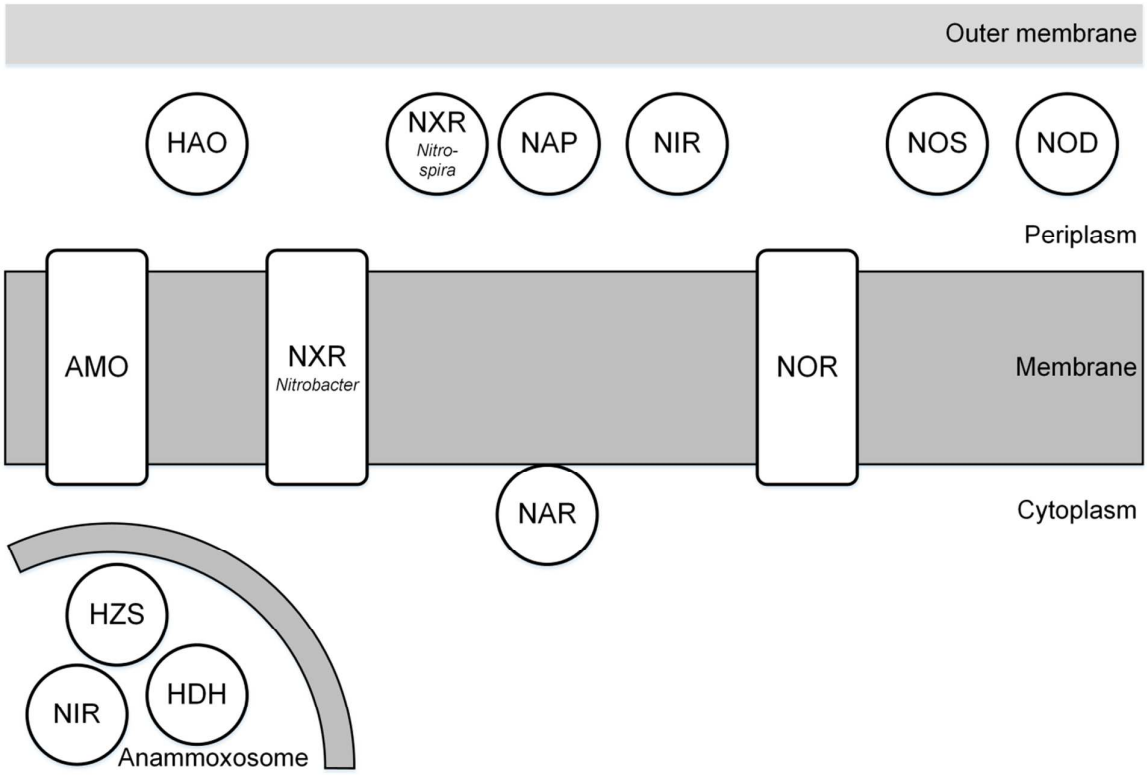

Figure 2 - Location of functional enzymes. The catalytic site of ammonia monooxygenase (AMO) and nitrite oxidoreductase (NXR) *Nitrobacter*, and nitrate reductase (NAR) are located in the cytoplasm. Hydrazine synthase (HZS), hydrazine dehydrogenase (HDH) and nitrite reductase (NIR) of anammox are located in the anammoxosome. Hydroxylamine dehydrogenase (HAO), nitrite oxidoreductase (NXR) *Nitrospira*, nitrate reductase (NAP), nitrite reductase (NIR), nitrous oxide reductase (NOS), nitric oxide dismutase (NOD) and the catalytic site of nitric oxide reductase (NOR) are located in the periplasm.

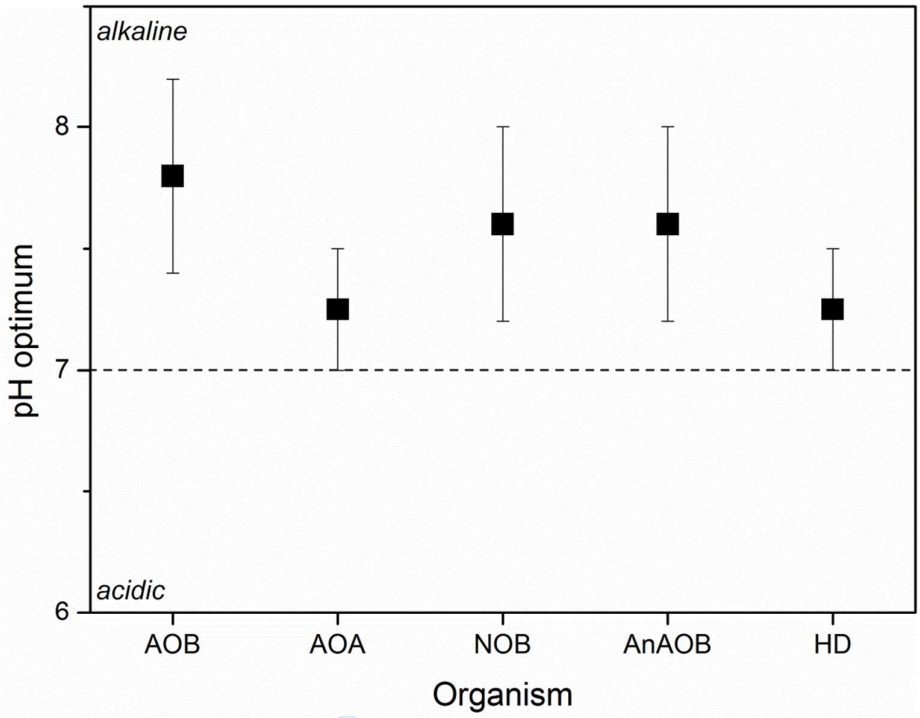

Figure 3 - Physiological pH optima of organisms reported in literature. Organisms: AOB, aerobic ammonia oxidizing bacteria; AOA, ammonia oxidizing archaea; NOB, nitrite oxidizing bacteria; AnAOB, anaerobic ammonia oxidizing bacteria; HD, heterotrophic denitrifiers. References are given in Table S1.

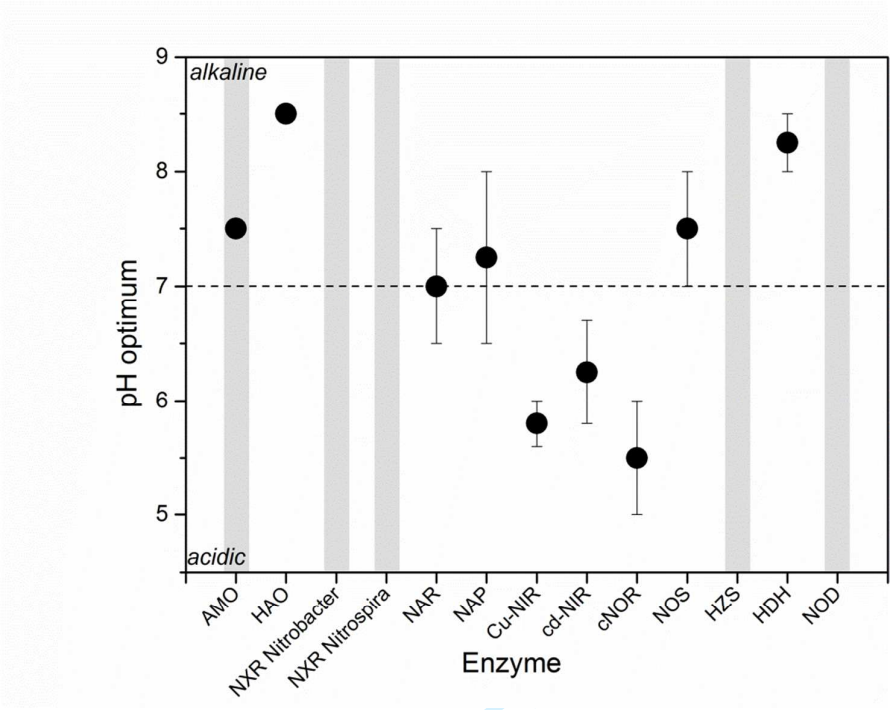

Figure 4 - pH optima of enzymes involved in N-conversion. Enzymes: ammonia monooxygenase (AMO), hydroxylamine dehydrogenase (HAO), nitrite oxidoreductase (NXR),nitrate reductase (NAR), periplasmic nitrate reductase (NAP), nitrite reductase (NIR), nitric oxide reductase (NOR), nitrous oxide reductase (NOS), hydrazine synthase (HZS), hydrazine dehydrogenase (HDH), and nitric oxide dismutase (NOD). Enzymes in gray shade (AMO, NXR *Nitrospira*, NXR *Nitrospira*, HZS and NOD) represent a lack of reported optimum pH. The optimum pH of AMO was referred to the particulate methane monooxygenase (pMMO). References are given in Table 1.

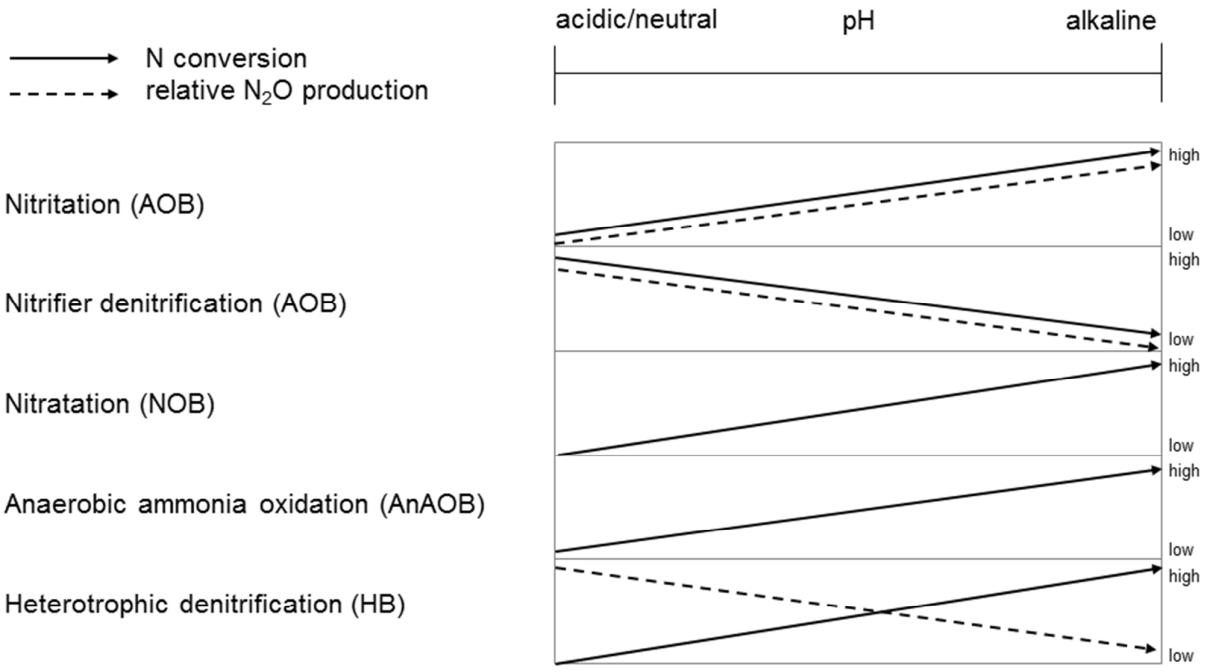

Figure 5 - Qualitative changes of N-conversion and N_2O production rates of different pathways with varying pH.